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In vitro antioxidant activity and polyphenol estimation of methanolic extract of endangered medicinal tree species, *Hildegardia populifolia* (Roxb.) Schott & Endl.

Saradha .M[1] and S. Paulsamy*[1]

*Corresponding author:

S. Paulsamy

[1]Department of Botany, Kongunadu Arts and Science College, Coimbatore-641 029.

Abstract

Antioxidant property of methanolic leaf and stem bark extracts of the endangered medicinal tree species, *Hildegardia populifolia* was evaluated by studying the contents of total phenolics, tannins and flavonoids, free radical scavenging activity using 1,1-diphenyl-2-picryl hydrozyl (DPPH), hydroxyl radical scavenging activity, reducing power activity, $ABTS^{\cdot+}$ assay and metal chelating activity. The results of the study revealed that both the parts studied were found to have potent antioxidant activity against DPPH, hydroxyl and $ABTS^{\cdot+}$ radicals with the IC^{50} value of 92.86 and 309.83 for methanolic extracts of leaf and stem bark respectively for DPPH radicals and 820.30 (leaf) and 456.71 (stem bark) for hydroxyl radicals. Therefore methanolic extracts of both leaf and stem bark of *H. populifolia* can be considered as a new potential source of natural antioxidants for pharmaceutical industries.

Keywords: Hildegardia populifolia, free radical scavenging activity (DPPH), hydroxyl radical scavenging activity, reducing power activity, $ABTS^{\cdot+}$, metal chelating activity

Introduction

Antioxidants, which scavenge active oxygen species (free radicals) are found in a variety of food stuffs and are commonly referred to as scavengers. Many antioxidants are plant based and play an important role in protecting plants against strong sunlight and live under severe oxygen stress and also in human health care measures (Badami *et al.,* 2003; Atawodi, 2005; Shyur *et al.,* 2005; Katalinic *et al.,* 2006). According to recent research, activated oxygen is thought to be a major factor in ageing, hardening of the arteries, diabetes, cancer and tissue injury of skin. Indeed approximately 90% of age related diseases are linked to activated oxygen.

Recently, there has been a considerable interest in finding natural antioxidant from plant materials to replace synthetic ones. Natural antioxidant substances are presumed to be safe since they occur in plant foods, and are seen as more desirable than their synthetic counter parts (Pratt, 1992). Typical compounds that exhibit antioxidative activity include vitamins, carotenoids, and phenolic compounds (Morris *et al.*, 1998; Klipstein *et al.,* 2000; Moeller *et al.,* 2000; Slattery *et al.,* 2000). Exploration of plant species for antioxidant property is most essential for human health care. Members of Sterculiaceae are reported to have more antioxidant property due to the presence of wide spectrum of phenlics and flavonoids (Basniwal *et al.,* 2009; Pattanaik and Parida, 2010; Borokini and Omotayo, 2012). In light of this fact, the endangered medicinal tree species, *Hildegardia populifolia* of the family, Sterculiaceae was selected for the evaluation of antioxidant property. It is the indigenous species of southern India distributed in the Eastern Ghats of Villupuram, Salem and Erode districts and Western Ghats of Coimbatore district of Tamil Nadu, and Anantapur, Chittur and Cuddapah districts of Andrpradesh (Pullaiah, 2000). The stem bark and leaves of these plants are used for the treatment of dogbite and malaria in the traditional medical practice of Tamil Nadu and Andra Pradesh (Varaprasad *et al.,* 2009).

Materials and Methods

Plant material

The plant material was collected from the campus of Forest Genetic Division, Tamil Nadu Forest Department, Bhavanisagar, Erode, Tamil Nadu, India.

Preparation of extracts

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The shade dried leaves and stem bark of the study species were made into fine powder of 40 mesh size using the pulvarizer separately. Hundred gram of the powder was filled in the filter paper and successively extracted by using 500mL methanol in soxhlet extractor for 8 to 10 hours (Gafner *et al.,*1985). Then the extract was filtered through Whatman No. 1 filter paper to remove all undissolved matter, including cellular material and other constituents that are insoluble in the extraction solvent.

Determination of total phenolic and tannin contents

The total phenolic content was determined according to the method described by Siddhuraju and Becker (2003). Aliquots of each extract were taken in test tubes and made up to the volume of 1 ml with distilled water. Then 0.5 ml of folin-ciocalteu phenol reagent (1:1 with water) and 2.5 ml of sodium carbonate solution (20%) were added sequentially in each tube. Soon after vortexing the reaction mixture, the test tubes were placed in dark for 40 min and the absorbance was recorded at 725 nm against the reagent blank. The analysis was performed in triplicate and the results were expressed as gallic acid equivalents (GAE). Using the same extract the tannins were estimated after treatment with polyvinyl polypyrrolidone (PVPP). Hundred mg of PVPP was weighed in a 100 x 12 mm test tube and to this 1.0 ml of distilled water and then 1.0 ml of tannin containing phenolic extract were added. The content was vortexed and kept in the test tube at 4 ºC for 4 h. Then the sample was centrifuged (3000 rpm for 10 min at room temperature) and the supernatant was collected. This supernatant has only simple phenolics other than tannins (the tannins would have been precipitated along with the PVPP). The phenolic content of the supernatant was measured, as monitored above and expressed as the content of non-tannin phenolics on dry matter. From the above results, the tannin content of the sample was calculated as follows:

Tannin (%) = Total phenolics (%) – Non-tannin phenolics (%).

Estimation of total flavonoid content

The total flavonoid content of samples was determined by following the modified colorimetric method of Zhishen *et al.* (1999). 0.5 ml extract was mixed with 2 ml of distilled water and subsequently with 0.15 ml of 5% $NaNO_2$ solution. After 6 min, 0.15 ml of 10% $AlCl_3$ solution was added and allowed to stand for 6 min, then 2 ml of 4% NaOH solution was added to the mixture. Immediately distilled water was added to bring the final volume to 5 ml, and then the mixture was thoroughly mixed and allowed to stand for another 15 min. Absorbance of the mixture was recorded at 510 nm versus prepared water blank. Rutin was used as a standard compound for the quantification of total flavonoid. All the values were expressed as milligram of rutin equivalent (RE) per gram of extract.

DPPH radical scavenging activity

The 2, 2-diphenyl-picryl-1-picryl-hydrazyl radical (DPPH) scavenging activity was measured according to the method of Blois (1958). Methanol extract of the samples at various concentrations (100, 150,200, 250 and 300 µg/mL, and 250, 300, 350, 400 and 450 µg/mL for leaf and stem bark respectively) was added separately to each 5mL of 0.1mM methanolic solution of DPPH and allowed to stand for 20min. Absorbance at 517nm using spectrophotometer was measured. BHT was used as standard. The corresponding blank reading was also taken and DPPH radical scavenging activity was calculated by using the following formula:

DPPH radical scavenging activity (%) = Control OD-Sample OD/Control OD x100

IC_{50} value is the concentration of the sample required to scavenge 50% DPPH free radical / OH^+ radical which has been determined by using the software SPSS v.16.

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity of methanolic extracts of leaf and stem bark of *H.populifolia* was determined by following the method of Zhao *et al.* (2007). Reaction mixture prepared for this experiment contained 500 µL of $FeSO_4$, 500µL of phenanthroline, 2.5mL of PO_4 buffer (pH 7.8), 500µL of H_2O_2 and respective concentrations of leaf samples at 650, 700,750, 800 and 850 µg/mL and stem bark samples at 300, 350, 400, 450 and 500 µg/mL and the reaction was started by adding H_2O_2. After incubation at room temperature for 5min, the absorbance at 536nm was measured. The hydroxyl radical scavenging activities were calculated similarly to that of DPPH radical scavenging activity.

Assay of Reducing Power

Reducing power assay was determined according to the method of Yildirim *et al.,* 2007. Different concentrations of methanolic extracts of leaf and stem bark of the study species (100, 150, 200, 250 and 300 µg/mL) were mixed with 2.5mL of 200mM sodium potassium ferric cyanide and incubated at 50^0C for 20 min. After adding 2.5mL of 10% trichloro acetic acid, the mixture was centrifuged at 3000rpm for 10min. The supernatant was taken out and immediately mixed with 5mL of distilled water and 0.5mL of 1% ferric chloride. After incubation for 10min. the absorbance was measured at 700nm. Higher absorbance of the reaction mixture indicates reductive potential of the extract.

Antioxidant activity by the $ABTS^{\bullet+}$ assay

The total antioxidant activity of the samples was measured by $ABTS^{\bullet+}$ [2,2-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid)] radical cation decolorization assay according to the method of Re *et al.* (1999). $ABTS^{\bullet+}$ was produced by reacting 7mM ABTS aqueous solution with 2.4 mM potassium persulfate in the dark for 12–16 h at room temperature. Prior to assay, this solution was diluted in ethanol (about 1:89 v/v) and equilibrated at 30 °C to give an absorbance of 0.700 ± 0.02 at 734 nm. The stock solution of the sample extracts was diluted such that after introduction of 10 µl

aliquots into the assay, which have been produced between 20% and 80% inhibition of the blank absorbance. After the addition of 1 ml of diluted ABTS solution to 10 µl of sample or trolox standards (final concentration 0–15 µM) in ethanol, absorbance was measured at 30 °C exactly 30 min after the initial mixing. Appropriate solvent blanks were also run in each assay. Triplicate determinations were made at each dilution of the standard, and the percentage inhibition was calculated from the blank absorbance at 734 nm and then it was plotted as a function of trolox concentration. The unit of total antioxidant activity (TAA) is defined as the concentration of trolox having equivalent antioxidant activity expressed as µmol/g sample extract on dry matter.

Metal chelating activity

The chelating of ferrous ions by leaf and stem bark methanolic extracts of *H. populifolia* was estimated by the method of Dinis *et al.* (1994). Briefly the extract samples (250 µl) were added to a solution of 2 mmol/L $FeCl_2$ (0.05 ml). The reaction was initiated by the addition of 5 mmol/L ferrozine (0.2 ml) and the mixture was shaken vigorously and left standing at room temperature for 10 min. Absorbance of the solution was then measured spectrophotometrically at 562 nm. The chelating activity of the extracts was evaluated using EDTA as standard. The results were expressed as mg EDTA equivalent/g extract.

Statistical analysis

All analyses were carried out in triplicate and the data were reported as means ± SD. The data were subjected to one way analysis of variance (ANOVA) and the significance of the difference between means was determined by Duncan's Multiple Range Test ($P<0.05$) using the statistical software Inc., Tulsa, OK, USA.

Results and Discussion

Total phenolic and tannin compounds

Phenolic compounds are known as powerful chain breaking antioxidants (Shahidi and Wanasundara, 1992) and they are very important plant constituents because of their scavenging ability by their hydroxyl groups (Hatano *et al.,* 1989). These compounds have an important role in stabilizing lipid oxidation and are associated with antioxidant activity (Yen *et al.,* 1993). The phenolic compounds may contribute directly to antioxidative action. It was suggested that polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans, when ingested upto 1gm daily from a diet rich in fruits and vegetables (Tanaka *et al.,* 1998). The tannin containing remedies are used as antioxidants (Kolickar *et al.,* 2008) in addition to member of other therapeutic uses. The total phenolic content of methanolic extracts of leaf and stem bark of the study species, *H. populifolia* is found to be 2254.26 and 1330.23 mg GAE/g extract respectively in terms of gallic acid equivalent (Table 1). Similarly, the tannin content of the study species *H. populifolia* gave higher yield of 855.81mg GAE/g extract for leaf and 254.26mg GAE/g extract for stem bark (Table 1).

Total flavonoid compound

Flavonoids present in food of plant origin are also potential antioxidants (Salah *et al.,* 1995; Van Acker *et al.,* 1996). Most of the beneficial effects of flavonoids are attributed to their antioxidant and chelating abilities (Hassig *et al.,* 1999). Studies have shown that certain flavonoids exhibit hypoglycaemic effect also (Ahmed *et al.,* 2000). The total flavonoid content of methanolic leaf and stem bark extracts was determined to be 0.11 and 0.29 mg RE/g extract respectively in terms of rutin equivalent (Table 1).

Free radical scavenging activity (DPPH method)

DPPH is a stable free radical that accepts an electron hydrogen radical to become a stable diamagnetic molecule (Dreosti, 2000). The decrease in absorbance of DPPH radical caused by antioxidants, because of reaction between antioxidant molecules and radicals, results in the scavenging of radical by hydrogen donation (Ogawa *et al.,* 2008). It is visually noticeable as a change in colour from purple to yellow. Hence, DPPH is usually used as a substrate to evaluate the antioxidative activity of antioxidants (Shah *et al.,* 2007; Chew *et al.,* 2009).

In the present study, the percentage of scavenging effect on the DPPH radical was increased with the increase in the concentration of both leaf and stem bark extracts from 100 to 300 µg/mL by showing the per cent inhibition from 51.16 at 100µg/mL to 89.1 at 300 µg/mL for leaf extract and for bark extract, they were 40.50% at 250 µg/mL and 67.77% at 450µg/mL. The IC_{50} values of leaf and bark extracts were 92.81 and 309.83 respectively over the standard, BHT, 28.12. From the results it is known that the species, *H. populifolia* possess hydrogen donating capabilities and does scavenging free radicals.

Hydroxyl radical scavenging activity

Scavenging of OH^+ is an important antioxidant activity because of its very high reactivity which can easily cross the cell membranes at specific sites, react with the biomolecules and further causes cell damage and finally cell death. Thus, removing of OH^+ is most important for the protection of living system (Yang *et al.,* 2008). Table 3 shows the OH^+ scavenging effects of leaf extracts of *H. populifolia* at different dose levels such as 650, 700,750, 800 and 850 µg/mL and bark extracts at 300, 350, 400, 450 and 500µg/mL. Both samples generally registered good hydroxyl radical scavenging activity and it was concentration dependant. Among them, the methanol extract of stem bark showed the highest OH^+ scavenging potential as the IC_{50} value, 456.7µg/mL against the IC_{50} value of leaf extracts, 820.3µg/mL. The ability of the *H. populifolia* extracts to quench hydroxyl radicals seems to be appreciable and hence the extracts are good scavengers of active oxygen species, thus reducing the rate of chain reaction.

Reducing power activity

Reducing power activity is based on the principle that substances which has reduction potential, react with potassium ferric cyanide (Fe^{+3}) to form potassium ferrous cyanide (Fe^{+2}), which then reacts with ferric chloride to form ferrous complex that has an absorption maximum at 700nm. Methanolic leaf and stem bark extracts of *H. populifolia* in which the reducing power of the extract was increasing with the increase in concentrations. The reducing power of both leaf and stem bark methanolic extracts of *H. populifolia* is comparable to that of standard L. ascorbic acid (Table 4). The reducing capacity of the study species may serve as a significant indicator of its potential antioxidant activity as reported for the species, *Rumex crispus* by Yildirim *et al.,* (2001).

$ABTS^{\cdot+}$ cation radical scavenging activity

$ABTS^{\cdot+}$, a protonated radical has characteristic absorbance maximum at 734nm which decreases with the scavenging of the proton radicals. $ABTS^{\cdot+}$ was generated by incubating ABTS [2,2-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid)] with potassium persulfate. The presence of chemical compounds in the tested extracts that inhibit the potassium persulfate activity may reduce the production of $ABTS^{\cdot+}$. The study for the species, *H. populifolia* reports that the methanolic leaf extract of the species registered the highest total antioxidant activity, 2143.79µmol/g (Table 5) followed by the bark extracts with 1804 µmol/g which indicates that both parts of the study species have considerable radical scavenging activity.

Table 1. Content of total phenolics, tannins and flavonoids content in methanolic leaf and stem bark extracts of *Hildegardia populifolia.*

Sample	Total phenolics (mg GAE/ g extract)	Total tannin (mg GAE/ g extract)	Total flavonoids (mg RE/ g extract)
Leaf	2254.26±10.74	855.81±9.30	0.11±0.01
Stem bark	1330.23±9.30	254.26±5.37	0.29±0.02

GAE - Gallic acid equivalent, RE – Rutin equivalent.

Table 2. Free radical scavenging activity (DPPH method) of methanol extracts of leaf and stem bark of *Hildegardia populifolia.*

Sl.No.	Leaf			Stem bark		
	Sample concentration (µg/ml)	Percentage activity	IC_{50} (µg/ml)	Sample concentration (µg/ml)	Percentage activity	IC_{50} (µg/ml)
1.	100	51.16^{e}± 0.40	92.86	250	40.50^{e}± 0.40	309.83
2.	150	72.09^{d}± 0.82		300	52.07^{cd}± 0.81	
3.	200	86.82^{bc}± 0.49		350	52.89^{c}± 1.63	
4.	250	87.60^{b}± 1.63		400	57.02^{b}± 1.22	
5.	300	89.15^{a}± 0.65		450	67.77^{a}± 0.81	

IC_{50} for the standard BHT = 28.12.
Values are expressed as mean±SD (n=6).
Values within the same column not sharing common superscript letters (a-e) differ significantly at $p<0.05$ by DMRT.

Table 3. Hydroxyl radical scavenging activity of methanolic extracts of leaf and stem barkof *Hildegardia populifolia.*

Sl.No.	Leaf			Stem bark		
	Sample concentration (µg/ml)	Percentage activity	IC_{50} (µg/ml)	Sample concentration (µg/ml)	Percentage activity	IC_{50} (µg/ml)
1.	650	17.20^{e}± 0.82	820.30	300	46.50^{d}± 0.79	456.71
2.	700	21.02^{d}± 0.49		350	48.41^{cd}± 0.62	
3.	750	38.85^{bc}± 0.65		400	49.04^{bc}± 0.54	
4.	800	40.76^{b}± 0.82		450	49.68^{b}± 0.87	
5.	850	45.85^{a}± 0.33		500	51.71^{a}± 0.38	

IC_{50} for the standard BHT = 28.69.
Values are expressed as mean±SD (n=6).
Values within the same column not sharing common superscript letters (a-e) differ significantly at p<0.05 by DMRT.

Table 4. Reducing power assay of methanol leaf and bark extract *Hildegardia populifolia*

Sl.No	Sample/StandardConcentration (µg/ml)	Absorbance at 700nm		
		Methanolic leaf extract	Methanolic stem bark extract	Standard L. ascorbic acid
1.	100	0.24^{e}±0.09	0.19^{e}±0.03	0.47^{e}±0.08
2.	150	0.36^{d}±0.06	0.24^{d}±0.09	0.56^{d}±0.02
3.	200	0.45^{bc}±0.08	0.30^{c}±0.06	0.61^{c}±0.05
4.	250	0.46^{b}±0.04	0.33^{b}±0.07	0.72^{b}±0.05
5.	300	0.49^{a}±0.08	0.39^{a}±0.02	0.85^{a}±0.03

Table 5. ABTS and metal chelating activity of leaf and stem bark extracts of *Hildegardia populifolia.*

Sample	Total antioxidant activity (µmol TE/g extract)	Metal chelating activity (mg EDTA/ g extracts)
Leaf	2143.79 ± 2.34	150.80 ± 2.01
Stem bark	1804 ± 4.68	176.77 ± 0.12

Total antioxidant activity (µmol equivalent trolox performed by using $ABTS^{\cdot+}$ radical cation).

Metal chelating activity

Iron is essential for life system because it is required for oxygen transport, respiration and activity of many enzymes. In complex systems such as food and food preparation many different mechanisms may contribute to oxidative process such as Fenton reaction, where transition metal ions play a vital role. Different reactive oxygen species might be generated and due to which various target structures like lipids, proteins, carbohydrates etc. can be affected. Therefore it is important to characterize the extracts by a variety of antioxidant assays (Halliwell, 1997). The chelating effect on ferrous ions by the methanolic leaf and stem bark extracts of *H. populifolia* is presented in Table 5. Both the samples exhibited the ability to chelate metal ions and among

them, the activity was found to be slightly lower for methanolic leaf extract of *H. populifolia* (150.80mg EDTA/g). Metal chelating capacity was significant as they reduced the concentration of the catalyzing transition metal in lipid peroxidation (Duh *et al.,* 1999). It was already reported that chelating agents which form σ bonds with a metal are effective as secondary antioxidants because they reduce the redox potential, there by stabilizing the oxidized form of the metal ion (Keowmaneechai and McClements, 2006). Antioxidants inhibit interaction between metal and lipid through the formation of insoluble metal complexes with ferrous ion. Hence, the data obtained for study species reveals that the extracts of both leaf and stem bark demonstrate an effective capacity for iron binding and hence the antioxidant property.

Conclusion

This research provides information about the antioxidant property of the leaf and bark parts of H. populifolia. Hence, it is identified that this species can be used as a source for the manufacturing of drugs of scavenging property. However, large scale in vivo studies are required to confirm the scavenging property before going for commercialization..

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